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Trichuris suis: A Secretory Chymotrypsin/Elastase Inhibitor with Potential as an Immunomodulator

M. L. Rhoads, R. H. Fetterer, D. E. Hill, and J. F. Urban, Jr.

Parasite Biology and Epidemiology Laboratory, Livestock and Poultry Sciences Institute, USDA, ARS, BARC, Beltsville, Maryland 20705

Rhoads, M. L., Fetterer, R. H., Hill, D. E., and Urban, J. F., Jr. 2000. *Trichuris suis:* A secretory chymotrypsin/elastase inhibitor with potential as an immunomodulator. *Experimental Parasitology* **95,** 36–44. A serine protease inhibitor, termed TsCEI, was purified from adult-stage *Trichuris suis* by acid precipitation, affinity chromatography (elastase–agarose), and reverse-phase HPLC. The molecular weight of TsCEI was estimated at 6.437 kDa by laser desorption mass spectrometry. TsCEI potently inhibited both chymotrypsin (K_i = 33.4 pM) and pancreatic elastase (K_i = 8.32 nM). Neutrophil elastase, chymase (mouse mast cell protease-1, mMCP-1), and cathepsin G were also inhibited by TsCEI, whereas trypsin, thrombin, and factor Xa were not. The cDNA-derived amino acid sequence of the mature TsCEI consisted of 58 residues including 9 cysteine residues with a molecular mass of 6.196 kDa. TsCEI displayed 48% sequence identity to a previously characterized trypsin/chymotrypsin inhibitor of *T. suis*, TsTCI. TsCEI showed 36% sequence identity to a protease inhibitor from the hemolymph of the honeybee *Apis mellifera*. Sequence similarity was also detected with the trypsin/thrombin inhibitor of the European frog *Bombina bombina*, the elastase isoinhibitors of the nematode *Anisakis simplex*, and the chymotrypsin/elastase and trypsin inhibitors of the nematode *Ascaris suum*. The inhibitors of *T. suis*, an intestinal parasite of swine, may function as components of a parasite defense mechanism by modulating intestinal mucosal mast cell-associated, protease-mediated, host immune responses.

Index Descriptors and Abbreviations: chymotrypsin (EC3.4.21.1) inhibitor; elastase (EC3.4.21.36) inhibitor; chymase inhibitor; cathepsin G inhibitor; nematode; *in vitro* cultivation; excretory/secretory products; *Apis mellifera*; honeybee; TsCEI, *Trichuris suis* chymotrypsin/elastase inhibitor; TsTCI, *T. suis* trypsin/chymotrypsin inhibitor; AmCI, *A. mellifera* cathepsin G/chymotrypsin inhibitor; BSTI, *Bombina bombina* skin trypsin/thrombin inhibitor ATI, *Ascaris suum* trypsin inhibitor; C/E-1, *A. suum* chymotrypsin/elastase inhibitor; AX-SPI-1,2,3, *Anisakis simplex* elastase isoinhibitors; pNA, paranitroanilide; TCA, trichloroacetic acid; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TFA, trifluoroacetic acid; DTT, dithiothreitol.

INTRODUCTION

The whipworm, *Trichuris suis*, burrows into the mucosal lining of the cecum and colon of its swine host and stimulates a stereotypical host response to gastrointestinal parasites that is characterized by mucosal mast cell hyperplasia, eosinophilia, and elevated levels of reaginic antibodies (Urban *et al.*, 1998). Mast cells release an array of potent chemical mediators including serine proteases (Schwartz and Austen, 1984). In particular, intestinal nematode infections upregulate the secretion of the serine protease chymase, a chymotrypsin-like protease predominantly expressed in intestinal mucosal mast cells (Wastling *et al.*, 1997) and implicated as a central modulator of inflammation through its ability to directly activate interleukin-1β (Mitzutani *et al.*, 1991), enhance epithelial cell permeability (Scudamore, 1998), and stimulate inflammatory cell recruitment (Walls *et al.*, 1993; He and Walls, 1998). Cathepsin G, a chymotrypsin-like serine protease, and the serine protease elastase are released by activated neutrophils and also function as modulators of the inflammatory process.

Because proteases play critical roles in a variety of immune cell processes, protease inhibitors have the potential

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to modulate these responses. Recently, we reported the secretion of a *T. suis* inhibitor active against the serine proteases trypsin and chymotrypsin and purified a 6.7-kDa trypsin/chymotrypsin-specific peptide inhibitor, TsTCI (Rhoads *et al.*, 2000). The present study describes the purification and characterization of a chymotrypsin/elastase-specific peptide inhibitor.

MATERIALS AND METHODS

Parasites. Adult male and female *T. suis* were removed with forceps from the mucosal surface of the cecum and colon of infected pigs. Parasites were rinsed consecutively with 0.85% NaCl and sterile Hanks' balanced salt solution (HBSS) and then incubated at 37°C for 1 h in sterile RPMI 1640 medium containing 500 units ml^{-1} penicillin, 0.5 μg ml^{-1} streptomycin, 1.25 μg ml^{-1} fungizone, and 350 μg ml^{-1} chloramphenicol. After a final rinse with HBSS to remove antibiotics, parasites were either homogenized or cultured *in vitro* as described (Rhoads *et al.*, 2000). Protein concentrations of the homogenates elastase and culture fluids were estimated by the spectrophotometric nucleic acid/protein analysis program (Warburg-Christian) provided with the Beckman 640 spectrophotometer.

Inhibitor assays. Inhibitor activity was assayed in 96-well plates by the inhibition of hydrolysis of synthetic peptide–paranitroanilide (pNA) substrates by their respective proteases. The assay consisted of 2.0 nM protease (except for factor Xa at 0.5 nM, human neutrophil elastase at 17 nM, cathepsin G at 220 nM, and chymase at 160 nM), 50 mM Tris/HCl buffer, pH 7.5, containing 0.15 M NaCl and 0.1% bovine serum albumin (BSA) and a 10-μl aliquot of *T. suis* samples (extract, culture fluids, column fractions, or purified peptide). Following a 30-min incubation, substrate (0.25 mM, except for elastase, cathepsin G, and chymase substrates at 1.0 mM) was added and the residual activity (mOD/min) was determined at 405 nm on a Vmax microplate kinetic reader (Molecular Devices). The total volume of the assay was 200 μl. Chymotrypsin (TLCK-treated, Sigma) was asayed with 3-carbomethoxypropionyl–L-arginyl–L-prolyl–L-tyrosine–pNA (Chromogenix S-2586, Diapharma, West Chester, OH); elastase (bovine pancreas, Calbiochem, La Jolla, CA) was assayed with Boc–alanyl–alanyl–prolyl–alanine–pNA (Calbiochem); elastase (human neutrophil, Calbiochem) was assayed with MeOSuc–ala–ala–pro–val–pNA (Calbiochem); trypsin (bovine pancreas, TPCK-treated, Sigma) was assayed with *N*-benzoyl–L-isoleucyl–L-glutamylglycyl–L-arginine–pNA (Chromogenix S-2222, Diapharma); cathepsin G (Calbiochem) and mMCP-1 (mouse mast cell protease-1, a chymase, kindly supplied by Dr. Hugh Miller, The University of Edinburgh, Scotland) were assayed with Suc–ala–ala–pro–phe–pNA (Calbiochem); thrombin (bovine plasma, Sigma) was assayed with H-D-phenylalanyl–L-pipecolyl–L-arginine–pNA (Chromogenix S-2238, Diapharma); and factor Xa (bovine plasma, Diapharma) was assayed with *N*-α-benzyloxycarbonyl–D-arginyl–L-glycyl–L-arginine–pNA (Chromogenix S-2765, Diapharma).

Trichloroacetic acid (TCA) precipitation. Fifty percent (w/v) TCA was added slowly to the *T. suis* soluble extract while stirring to a final concentration of 2.5%. This solution was left at room temperature for 30 min and then centrifuged at 17,300*g* for 10 min. The supernatant (TCA-soluble fraction) was dialyzed extensively with multiple changes of 50 mM Tris buffer, pH 7.6.

Elastase–agarose affinity chromatography. Elastase (porcine pancreas) was covalently coupled to AminoLink Plus Coupling Gel (4% beaded agarose, Pierce, Rockford, IL) according to manufacturer's instructions. Briefly, 4 ml of elastase (2× crystallized aqueous suspension, Sigma) dissolved in coupling buffer (0.1 M sodium phosphate, 0.15 M NaCl, pH 7.2) at a concentration of 1.5 mg/ml was incubated overnight with the gel matrix. Following immobilization, the gel was equilibrated with 50 mM Tris buffer, pH 7.6, containing 0.3 M NaCl and 10 mM $CaCl_2$. The dialyzed *T. suis* TCA-soluble fraction (routinely 15–20 mg) was loaded onto the column, the flow rate was stopped, and the sample was allowed to incubate with the elastase–agarose for approximately 10 min. The column was then eluted with equilibration buffer and fractions were monitored at 280 nm. When the absorbance returned to baseline, bound proteins were eluted with 0.01 M HCl; 0.5-ml fractions were collected and assayed for inhibitor activity. Active fractions were combined and lyophilized.

Reverse-phase HPLC. The lyophilized sample from the affinity column was resuspended in 0.1% trifluoroacetic acid (TFA) and separated on a Jupiter C-18, 5-μm, 250 × 4.6-mm steel column. The mobile phases consisted of A (0.1% aqueous TFA) and B (0.13% TFA in 70% aqueous acetonitrile). The sample (100 μl) was applied and eluted at a flow rate of 1 ml/min with 85% A for 5 min, followed by a linear gradient from 85% A to 100% B in 30 min; eluent was monitored at 214 nm. Fractions were assayed for elastase (bovine pancreas) inhibitor activity, active fractions were pooled and rotary evaporated, and the resulting sample was reconstituted in water. The peptide concentration was estimated by absorbance at 205 nm (Stoschek, 1990), and the specificity of the purified inhibitor was determined with chymotrypsin, neutrophil elastase, chymase, cathepsin G, trypsin, thrombin, and factor Xa. The purified inhibitor was termed TsCEI.

Apparent dissociation constants. The apparent equilibrium dissociation inhibitory constant (K_i) was determined for TsCEI with elastase (bovine pancreas) and chymotrypsin. Using the described assay, ratios of inhibited velocity (V_i) to the uninhibited velocity (V_0) were plotted against the concentration of TsTCI as determined by quantitative amino acid analysis. Data were fitted to an equation for tight-binding inhibitors (Bieth, 1974) and the K_i values were determined.

Laser desorption mass spectrometry (LDMS). The molecular mass of TsCEI was determined by matrix-assisted laser desorption mass spectrometry. The peptide was mixed with saturated α-cyano-4-hydroxycinnamic acid matrix solution and pulsed with a nitrogen laser (337 nm) at 20,000 V.

Amino terminal and internal sequence analysis. Following Edman degradation, the N-terminal sequence of TsCEI was determined on an Applied Biosystems Procise liquid-pulse protein sequenator. Internal sequences were determined by subjecting TsCEI to Lys C protease digestion (2 to 4 pmol Lys C, 100 mM Tris, pH 8.8, 2 M urea, 2.2 mM DTT, and 5 mM iodoacetamide) at 37°C for 24 h. The peptide digest was fractionated by HPLC and selected peaks were subjected to amino acid sequencing as described above. Peaks chosen for sequencing were also analyzed by LDMS to confirm mass estimations.

Isolation of mRNA and cDNA cloning. Total RNA was extracted from adult *T. suis* by the method of Zarlenga and Gamble (1987). First

strand cDNA was generated by RT-PCR using 20 μg of total RNA and 50 μM oligo(dT)$_{16}$ as previously described (Lu *et al.*, 1998). The original primer sequences, designed from the amino acid sequence data obtained as described above, were degenerate with inosine used as the third base in each codon. Amplification of inhibitor-specific cDNA by PCR was accomplished using the first strand cDNA as a template and oligonucleotide primers based upon the exact sequence of the molecule. Each primer (Table I) contained a *Hin*dIII or *Eco*RI restriction site at the 5′ end to allow subcloning in a known orientation for double-stranded DNA sequencing. Three additional bases (ACA) or a GCCG clamp was added to ensure polymerization through the restriction sites. Amplification of the 5′ end of the inhibitor cDNA was accomplished using the modified conserved nematode 22-bp splice leader (SL-1) as the sense primer (Blaxter *et al.*, 1994) and antisense primer based upon the peptide sequence ACTMQC. The 3′ end of the cDNA was confirmed using oligonucleotides based upon the peptide sequence EQYTSC as the sense primer and oligo(dT)$_{16}$ as the antisense primer. The PCR contained 200 pM of each primer, 5 units of AmpliTaq DNA polymerase, and 200 μM dNTP (Gene Amp Kit, Perkin–Elmer, Branchburg, NJ). The reaction mixture was heated to 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 63°C for 1 min, and 72°C for 45 s. The PCR products were separated on 1.5% agarose gels (FMC Bioproducts, Rockland, ME), isolated using glass milk (USBioclean MP Kit, USB, Cleveland, OH), cloned into the pBluescript plasmid vector (Stratagene), and sequenced by the dideoxy chain-terminator method (T7 Sequenase v.2.0 Kit, Cleveland, OH). The full-length cDNA sequence was obtained by PCR using the modified SL-1 sequence as the sense primer and antisense primers derived from sequence information determined above from regions near the poly(A)tail. PCR reaction conditions, cloning, and sequencing were as described.

RESULTS

Elastase inhibitor activity was present in *T. suis* soluble extract and excretory–secretory products (Fig. 1); elastase was inhibited in a concentration-dependent manner. The extract contained 2.0 units and the culture fluids 0.28 units of elastase inhibitor activity (a unit is defined as the micrograms of *T. suis* protein required to inhibit elastase activity by 50%), indicating an enrichment of the inhibitor in culture fluids.

TABLE I

Oligonucleotide Primers Used in RT-PCR for Amplification of cDNA Containing *Trichuris suis* Serine Protease Inhibitor TsCEI

5′ Sense primers
Modified 5′ SL-1:
GCCGGAATTCGGTTTAATTACCCAAGTTGGAG
EQYTSC: ACAGAATTCGAACAGTATACATCCT
3′ Antisense primers
Oligo(dT): GCCGAAGCTT–Oligo(dT)$_{16}$
ACTMQC: ACAAAGCTTACATTGCATTGTACA

*Note. Eco*RI and *Hin*dIII restriction sites are underlined.

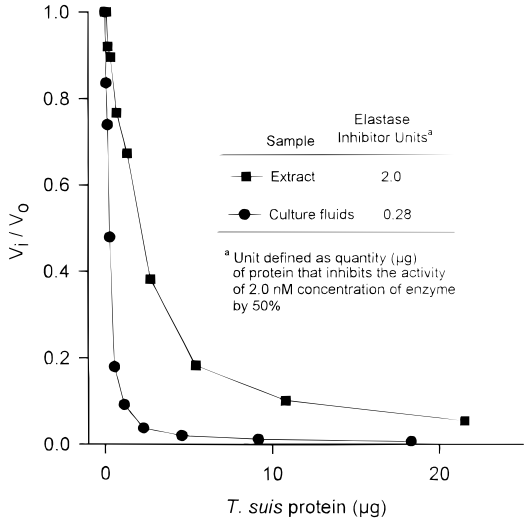

FIG. 1. Concentration-dependent inhibition of elastase by *T. suis* soluble extract and culture fluids. The relationship of the ratio of inhibited enzyme velocity (V_i) to uninhibited velocity (V_0) and the micrograms of protein of *T. suis* extract (solid squares) and culture fluids (solid circles) is shown. The data were fitted to a polynomial equation and the micrograms of *T. suis* extract and culture fluids at 50% enzyme inhibition were determined; inhibitor units are shown in the inset.

Following TCA precipitation of the *T. suis* soluble extract, the elastase inhibitor remained in solution. The elastase inhibitor present in the TCA-soluble fraction bound to the elastase–agarose column (little or no activity was present in the unbound fraction) and was eluted with 0.01 M HCl; this HCl-eluted fraction also inhibited chymotrypsin. Reverse-phase HPLC of the elastase–agarose-bound fraction resulted in one major protein peak (elution time of 22 min). (Fig. 2); this protein peak coincided with peaks of both elastase and chymotrypsin inhibition. Analysis of this peak by laser desorption mass spectrometry identified one component with the molecular mass estimated at 6.437 kDa (Fig. 3). The purified peptide was termed TsCEI.

The *T. suis* purified peptide inhibited chymotrypsin with a K_i estimated at 33.4 pM and pancreatic elastase with a K_i estimated at 8.32 nM (Fig. 4). Partially purified TsCEI (obtained by reverse-phase separation of the TCA-soluble fraction) also inhibited neutrophil elastase, cathepsin G, and the chymase mMCP-1 (Fig. 5). TsCEI did not inhibit trypsin, thrombin, or factor Xa (data not shown).

A 25-residue amino-terminal sequence (NH_2-ETQCGPNEQYTSCGSACPLTCEDIK) and a 13-residue internal sequence (ACTMQCVPGCFCK) were obtained by automated Edman degradation analysis. Oligonucleotide probes designed from these sequences were used for PCR cloning of

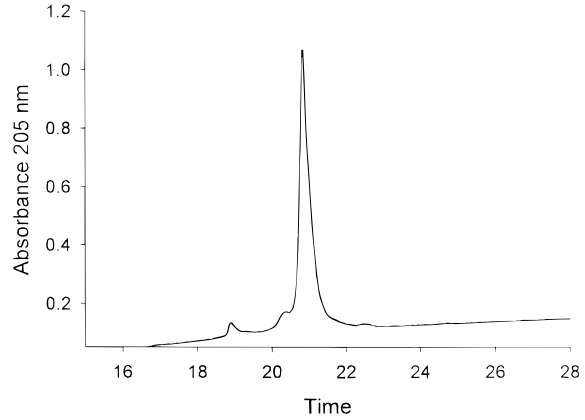

FIG. 2. Reverse-phase HPLC elution profile of the elastase–agarose-bound fraction of *T. suis* extract.

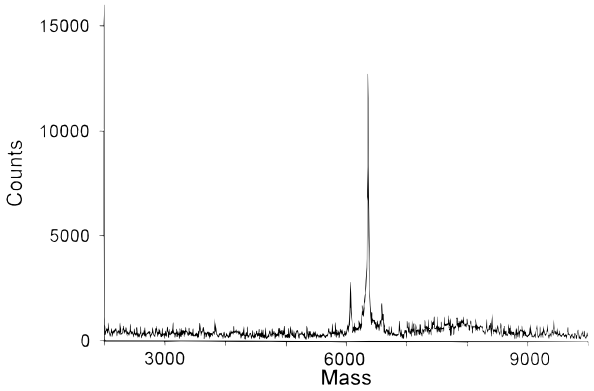

FIG. 3. Laser desorption mass spectrometry profile of the fraction from reverse-phase HPLC.

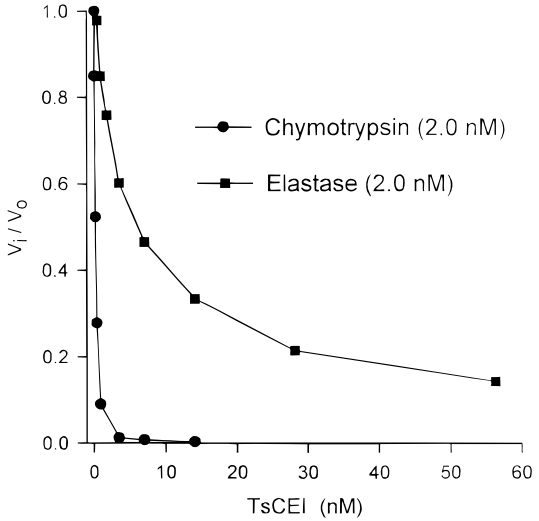

FIG. 4. Concentration-dependent inhibition of chymotrypsin (circles) and pancreatic elastase (squares) by TsCEI. The ratio of inhibited enzyme velocity (V_i) to uninhibited velocity (V_0) was plotted against the nanomolar concentration of TsCEI determined by quantitative amino acid analysis. Data were fitted to an equation for tight binding inhibitors (Bieth, 1974) and the apparent dissociation constants were estimated.

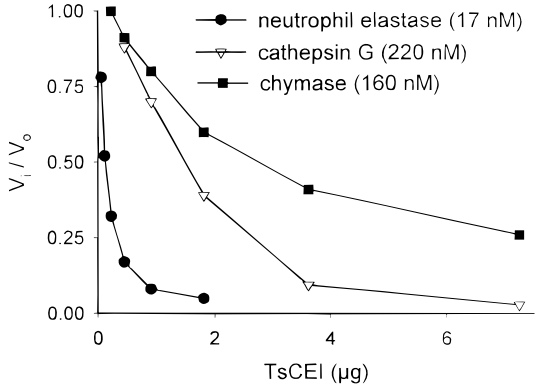

FIG. 5. Concentration-dependent inhibition of neutrophil elastase (circles), cathepsin G (triangles), and mMCP-1, a chymase (squares), by partially purified TsCEI. The ratio of inhibited enzyme velocity (V_i) to uninhibited velocity (V_0) was plotted against the micrograms of protein of partially purified TsCEI.

TsCEI (Table I). The complete cDNA coding sequence of TsCEI (GenBank Accession No. AF176644) is shown in Fig. 6. The 345-bp nucleotide sequence of TsCEI contained a single translational open reading frame extending from the initiator methionine (positions 64–66) to a stop codon, TGA, at positions 283–289. The open reading frame encoded 73 amino acids and a 5′ untranslated region of 63 nucleotides that included the modified SL-1 sequence at the 5′ terminal end. The initiator codon was flanked by sequences conforming to the Kozak translation initiation prediction (Kozak, 1986). The 5′ end contained a putative signal sequence of 15 amino acid residues that was adjacent to a potential signal sequence cleavage site (denoted by the forward slash) (Von Heijne, 1986) between residues −1 and +1, which gave an amino-terminal glutamic acid and an N-terminal 25-residue sequence identical to that determined by automated Edman degradation. The mature polypeptide consisted of 58 amino acids with a calculated molecular weight of 6.196 kDa.

TsCEI showed 48% sequence identity to a previously characterized trypsin/chymotrypsin inhibitor of *T. suis*, termed TsTCI (Rhoads *et al.*, 2000) (Fig. 7). The TsCEI sequence was screened against all nonredundant protein sequence databases maintained by the National Center for

FIG. 6. Nucleotide sequence and deduced amino acid sequence of TsCEI precursor. The beginning of the mature protein (end of the signal peptide) is marked by a forward slash. Underlined sequences are sense primers used to discern the 5′ end; double underlined sequences are antisense primers used to discern the 3′ end of the molecule. Single asterisk denotes the putative initiator methionine codon (Kozak, 1986); double asterisk denotes the stop codon. There is a 5′ untranslated region (63 nucleotides) that includes the 22-bp SL-1 primer sequence.

Biotechnology Information using Gapped BLAST 2.0. TsCEI showed 36% sequence identity with an inhibitor from the hemolymph of the honeybee *A. mellifera* (SWISS-PROT locus 4699856) (Bania *et al.*, 1999) with the introduction of a 13-residue gap in the *A. mellifera* sequence (Fig. 7). Sequence similarity was also detected with the trypsin inhibitor of the European frog *Bombina bombina* and the elastase isoinhibitors (AX-SPI-1-3) and the chymotrypsin/elastase (C/E-1) and trypsin (ATI) inhibitors of the nematodes *Anisakis simplex* and *Ascaris suum*, respectively, and again identity with the TsCEI sequence was maximized by the addition of a 12- to 14-residue gap in the sequences of these inhibitors (Fig. 7). In addition, the 11-residue sequence CTMQC-VPGCFC exhibited similarity to sequences of the inhibitors of *A. mellifera, B. bombina, A. simplex*, and *A. suum* that contain the residues that interact with the catalytic site of their inhibited enzymes (Fig. 8). These similarities include the positions of four cysteine residues, two of which form the boundaries of the reactive site (Grasberger *et al.*, 1994; Hawley *et al.*, 1994; Huang *et al.*, 1994). By analogy to the reported reactive sites of the *A. mellifera* (Bania *et al.*, 1999) and *A. suum* (Peanasky *et al.*, 1987) inhibitors, the sequence CTMQC of TsCEI is suggested as its reactive site and is identical to that of *A. mellifera*. The suggested reactive site of TsTCI was CTRQC.

DISCUSSION

Trichuris suis adult parasites express two distinct, low-molecular-weight, serine protease inhibitors; these inhibitors are released by worms maintained *in vitro*. One of the inhibitors, TsTCI, was purified and characterized in a previous study (Rhoads *et al.*, 1999). This 6.7-kDa protein inhibited trypsin (K_i = 3.1 nM) and chymotrypsin (K_i = 24.5 nM) and contained a region (46 amino acids) with 36% sequence identity to the honeybee *A. mellifera* inhibitor AmCI; no significant sequence identity was detected between TsTCI and any other previously identified serine protease inhibitors. In the present study, a 6.2-kDa protease inhibitor, TsCEI, was purified. The enzyme specificity of TsCEI was distinct from that of TsTCI, inhibiting both chymotrypsin (K_i = 33.4 pM) and pancreatic elastase (K_i = 8.32 nM); neutrophil elastase, cathepsin G, and chymase were also inhibited. TsCEI and TsTCI showed 48% sequence identity to each other. TsCEI displayed 36% sequence identity to the honeybee inhibitor. In contrast to TsTCI, sequence similarity was displayed between TsCEI and the serine protease inhibitors of the parasitic nematodes *A. simplex* and *A. suum*, as well as a protease inhibitor from the skin secretions of the European frog *B. bombina* (Mignogna *et al.*, 1996). The *A. suum*

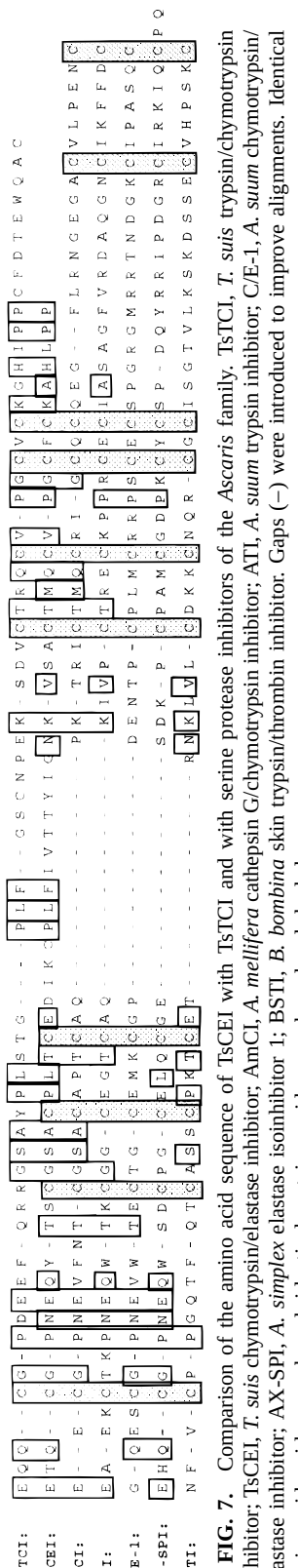

FIG. 7. Comparison of the amino acid sequence of TsCEI with TsTCI and with serine protease inhibitors of the *Ascaris* family. TsTCI, *T. suis* trypsin/chymotrypsin inhibitor; TsCEI, *T. suis* chymotrypsin/elastase inhibitor; AmCI, *A. mellifera* cathepsin G/chymotrypsin inhibitor; ATI, *A. suum* trypsin inhibitor; C/E-1, *A. suum* chymotrypsin/elastase inhibitor; AX-SPI, *A. simplex* elastase isoinhibitor 1; BSTI, *B. bombina* skin trypsin/thrombin inhibitor. Gaps (–) were introduced to improve alignments. Identical amino acid residues are boxed; identical cysteine residues are boxed and shaded.

inhibitors (five chymotrypsin/elastase and three trypsin isoinhibitors) (Grasberger *et al.*, 1994; Huang *et al.*, 1994), the *A. simplex* inhibitors (three elastase isoinhibitors) (Lu *et al.*, 1998), the inhibitor from the frog *B. bombina* (Mignogna *et al.*, 1996), and the inhibitor from the honeybee *A. mellifera* (Bania *et al.*, 1999) appear to belong to a novel family of protease inhibitors, having no sequence identity with the other well-characterized serine protease inhibitor families. The inhibitors of this restricted family are characterized as small proteins (60–65 residues) containing five disulfide bonds (formed by 10 cysteine residues present in a distinct pattern), two of which form the boundaries of the reactive site (at the P_3 and P_2 positions) (Huang *et. al.*, 1994; Grasberger *et al.*, 1994) (Figs. 7 and 8). The complete sequence of TsCEI consisted of 58 residues with 9 cysteine residues; TsTCI consisted of 61 residues with 8 cysteine residues. There was no pattern in the location of the cysteine residues between TsCEI and TsTCI; neither inhibitor contained the distinctive cysteine residue pattern associated with the *Ascaris* inhibitor family (Fig. 7). Furthermore, the position of the reactive site residues of TsCEI and TsTCI was not similar to that of the members of this family. Yet, TsCEI and TsTCI contained an 11-residue region that displayed similarities with the regions that contain the reactive sites of the *Ascaris* family of inhibitors (Fig. 8). TsCEI and TsTCI thus appear to be only distantly related to the *Ascaris* family of inhibitors.

The release *in vitro* of serine protease inhibitors by *T. suis* suggests that the targets for these inhibitors may be host proteases rather than endogenous parasite proteases. This is supported by the fact that both metallo- (Hill *et al.*, 1993) and cysteine (Hill and Sakanari, 1997) proteases, but no serine proteases have been detected in *T. suis*. The target host proteases for the *T. suis* inhibitors have yet to be identified. The ability of TsCEI to inhibit neutrophil elastase and cathepsin G, which are released by activated neutrophils and macrophages, as well as the chymase mMCP-1, which is released by activated mast cells, suggests that *T. suis* might utilize the secreted protease inhibitors to evade host attack. The mast cell-derived proteases, consisting of both tryptases and chymases, exist in multiple forms (Reynolds *et al.*, 1990; Stevens *et al.*, 1999) and are expressed in a cell- and/or tissue-specific fashion (Stevens *et al.*, 1994), suggesting highly specialized functions (Hunt and Stevens, 1995) that have yet to be fully elucidated. Significantly, mast cells appear to play a critical role in the expulsion of parasites from the gastrointestinal tract and thus are an important component in the host defense against parasitic infections (Miller, 1984, 1996). A mechanism that interferes with mast cell mediators could significantly contribute to parasite survival in the host.

Inhibitor	Amino acid sequence											
	P_3	P_2	P_1	P_1'	P_2'							
TsCEI:	C^{42}	T	M	Q	C^{46}	V	-	P	G	C	F	C^{52}
TsTCI:	C^{36}	T	R	Q	C^{40}	V	-	P	G	C	V	C^{46}
AmCI:	C^{28}	T	M	Q	C^{32}	R	-	I	G	C	Q	C^{38}
BSTI:	C^{30}	D	K	K	C^{34}	N	-	Q	R	C	G	C^{40}
ATI:	C^{29}	T	R	E	C^{33}	K	P	P	R	C	E	C^{40}
C/EI-1:	C^{29}	P	L	M	C^{33}	R	R	P	S	C	E	C^{40}
AX-SPI-1:	C^{27}	P	A	M	C^{31}	G	D	P	K	C	Y	C^{38}
AX-SPI-2,3:	C^{27}	A	T	I	C^{31}	G	E	P	K	C	Y	C^{38}

FIG. 8. Comparison of amino acid sequences of reactive site regions of protease inhibitors from *T. suis, A. mellifera, B. bombina, A. simplex*, and *A. suis*. TsCEI, *T. suis* chymotrypsin/elastase inhibitor; TsTCI, *T. suis* trypsin/chymotrypsin inhibitor; AmCI, *A. mellifera* cathepsin G/chymotrypsin inhibitor; BSTI, *B. bombina* skin trypsin/thrombin inhibitor; AX-SPI-1,2,3, *A. simplex* elastase isoinhibitors; C/E-1, *A. suum* chymotrypsin/elastase inhibitor; ATI, *A. suum* trypsin inhibitor. Bold letters indicate the reactive site residues. The residues around the reactive site peptide bond of the inhibitor that combine with the catalytic site of the enzyme in a substrate-like manner are designated P_3 to P_2'. The peptide bond is between residues P_1 and P_1'. P_1–P_1' residues were determined for AmCI and ATI by partial proteolytic cleavage at the reactive site by the cognate protease (Bania *et al.*, 1999; Peanasky *et al.*, 1987). Gaps were introduced to emphasize alignment of the cysteine residues (marked |).

The ability of protease inhibitors to function as effector molecules of immunomodulation is demonstrated by the regulatory role of endogenous α_1-antichymotrypsin that tightly controls the inflammatory process of mammals by inactivating the proteolytic activity of chymase and cathepsin G (Laine *et al.*, 1982). In addition, α_1-antichymotrypsin displays potent antiinflammatory properties both *in vitro* (Carney *et al.*, 1998) and *in vivo* (Murohara *et al.*, 1995; Scalia *et al.*, 1995; Hook *et al.*, 1995). Another serine protease inhibitor shown to affect mammalian immune system mediators is the Bowman–Birk inhibitor (BBI) isolated from soybeans and other legumes. BBI has been shown to have potent anticarcinogen and antiinflammatory activities in a number of *in vivo* and *in vitro* model systems (review, Kennedy, 1998); these biological activities have been linked to the chymotrypsin inhibitory activity of BBI. BBI has been shown to directly inhibit a number of modulators of the inflammatory process including cathepsin G and chymase (Larionova *et al.*, 1994; Ware *et al.*, 1997). In addition, BBI prevented both the influx of PMN leukocytes into regions of inflammation as well as their production of active oxygen species (Frenkel *et al.*, 1987).

Serine protease inhibitor-mediated modulation of host immune effector systems is a strategy used by viruses to inhibit host inflammatory responses to infection (Ray *et al.*, 1992; Macen *et al.*, 1993). Viral serine protease inhibitors were shown to contribute significantly to viral pathogenesis by interacting with host cellular components involved in the regulation of inflammation; specifically, a viral inhibitor was shown to prevent the activation of interleukin-1β, a critically important cytokine mediator of inflammatory responses (Ray *et al.*, 1992).

The severe clinical aspects of *T. suis* infections such as mucohemorrhagic enteritis have been associated with worm-induced suppression of mucosal immunity to intestinal tract resident bacteria (Mansfield and Urban, 1996). Concurrent infections with *T. suis* were shown to enhance the ability of opportunistic bacteria to multiply and cause disease and pathology; pathologic changes in the colon were noted both at sites of worm attachment and at distant sites. This generalized colon pathology can be explained by the strong and polarized type 2 immune response that is evoked by *T. suis* and other gastrointestinal parasites, restricting immune flexibility and thus allowing exploitation by opportunistic infections (Urban *et al.*, 1998). Additionally, the localized modulation of host immune effector systems by the *T. suis*-released inhibitors could also contribute to increased bacterial multiplication and pathology.

In summary, *T. suis* may synthesize and release multiple

serine protease inhibitors with distinct specificities for chymotrypsin-, trypsin-, and elastase-like enzymes as effector molecules of immunomodulation of the host mucosal mast cell-associated immune responses. Downregulation of host immune reactions by these parasite protease inhibitors may represent a critical mechanism for parasite survival and in addition, enhance the long-term survival of the host by modulating potentially immunopathogenic inflammatory responses. The *T. suis*-released serine protease inhibitors may also contribute to the decrease in host resistance to opportunistic pathogens, a direct and potentially lethal consequence of *T. suis* infections. In addition, these inhibitors could act as effective immunomodulators in the treatment of various gastrointestinal or other inflammatory diseases or serve as models in the design of effective synthetic prophylactic compounds.

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